Genetic modifiers of β-thalassemia

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As the defective genes for more and more genetic disorders become unravelled, it is clear that patients with apparently identical genotypes can have many different clinical conditions even in simple monogenic disorders. β thalassemia occurs when there is a deficiency in the synthesis of β globin chains. The clinical manifestations of β thalassemia are extremely diverse, spanning a broad spectrum from severe anemia and transfusion-dependency to the asymptomatic state of thalassemia trait. The remarkable phenotypic diversity of the β thalassemias is prototypical of how a wide spectrum of disease severity can be generated in single gene disorders. The most reliable and predictive factor of disease phenotype is the nature of the mutation at the β globin locus itself. However, relating phenotype to genotype is complicated by the complex interaction of the environment and other genetic factors at the secondary and tertiary levels, some implicated from family studies, and others, as yet unidentified. This article reviews the clinical and hematologic diversity encountered in β thalassemia with an overview of the modifier genes that moderate their disease expression.

Key words: β thalassemia, modifier genes.

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The thalassemias refer to a diverse group of hemoglobin disorders characterized by a reduced synthesis of one or more of the globin chains (α, β, γ, δβ, γδβ, δ and εγδβ).[1] β thalassemia occurs when there is a deficiency of β globin; typically, it is caused by a direct down-regulation in the synthesis of structurally normal β chains. However, a thalassemia phenotype can also arise from structural β chain variants if they are synthesized at a reduced rate, e.g. Hb E (β26 Glu→Lys). Alternatively, the variants are produced at a normal rate but are so unstable that they are rapidly destroyed giving rise to a functional deficiency. The former group of variants is also referred to as β thalassemic hemoglobinopathies. The hyperunstable β chain variants act in a dominant negative fashion, causing a disease phenotype even when present in a single copy, and hence, have been referred to as dominantly inherited β thalassemia.[2] In contrast, typical β thalassemia is inherited as a haploinsufficient Mendelian recessive disease. The thalassemias are the commonest monogenic disorders in the world and globally it is estimated that there are 270 million carriers, of which 80 million are carriers of β thalassemia. Archeological evidence in the Mediterranean region suggests that the disease was present as far back as the Neolithic period.[3] More than 200 mutations affecting the β globin gene are now known to result in a phenotype of β thalassemia. The most common forms are those that are prevalent in the malarial tropical and sub-tropical regions where a few mutations have reached high gene frequencies because of the protection they provide against malaria. In these countries in which β thalassemia is prevalent, a limited number of alleles (4 to 5) account for 90% or more of the β thalassemias, such that a focused molecular diagnostic approach can be undertaken.[4] The epidemiology of the disease, however, is changing due to a fall in total birth rate, prevention programs and recent population movements. In Northern Europe, including the UK, the US and Australasia, due to recent population migration, thalassemia has become an important part of clinical practice, and in these regions a much wider spectrum of β thalassemia mutations can be encountered. Furthermore, with improved clinical care and increasing survival of affected individuals, complications such as chronic liver disease, thrombosis and pulmonary hypertension are being increasingly recognized in the older patients.

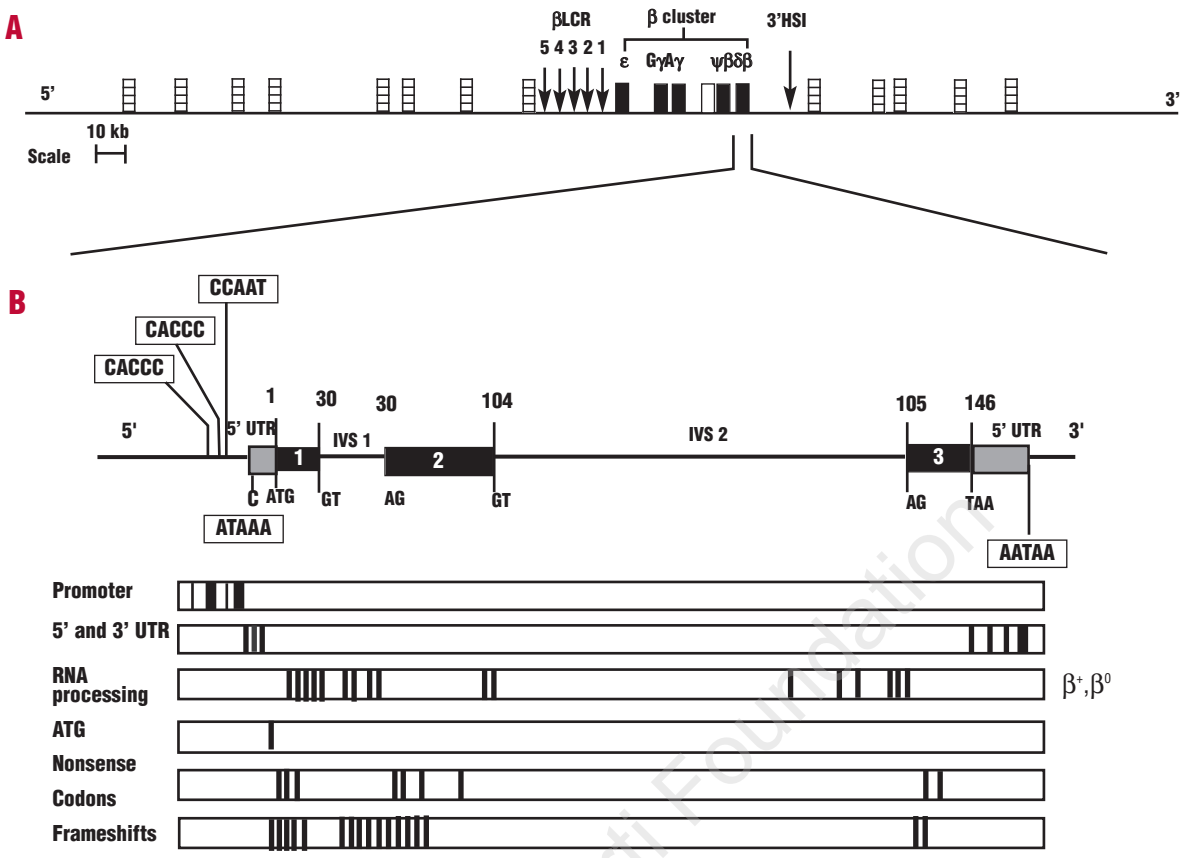

Figure 1. A. The β globin gene cluster and its flanking regions on chromosome 11p. The ε, ^{G}γ, ^{A}γ, δ and β globin genes are indicated as gray boxes. The 5' hypersensitive sites (1 to 5) which comprise the βLCR and the 3' hypersensitive sites (3'HS1) are shown as vertical arrows. Hatched boxes represent the olfactory receptor genes. B. General structure of the β globin gene with the 3 exons (gray boxes) and the 2 intervening sequences (IVS1 and IVS2). Conserved sequences (detailed in text) are indicated. The different classes of point mutations causing β thalassemia are shown below the β globin genes.

The β globin gene

β globin is encoded by a structural gene found in a cluster with the other β-like genes spanning 70 Kb on the short arm of chromosome 11 (11p15.4) (Figure 1). The cluster contains five functional genes, 5ε ^{G}γ-^{A}γ-ψβ, δ-β3', which are arranged in the order of their developmental expression. Upstream of the entire β globin complex is the locus control region (LCR), which is essential for the expression of all the genes in the complex. This region consists of five DNase 1 hypersensitive (HS) sites (designated HS1 5) distributed between 6 and 20 Kb 5' of the ε gene. There is one other hypersensitive site ~20 Kb 3' to the β gene. The two extreme HS sites flanking the β complex have been suggested to mark the boundaries of the β globin gene domain. The β globin complex is embedded in a cluster of olfactory receptor genes (ORG), part of the family of ~1000 genes that are widely distributed throughout the genome, and expressed in the olfactory epithelium.[5]

The general structure of the β globin gene is typical of the other globin loci[6,7] (Figure 1B). The genomic sequence spans 1600 bp and codes for 146 amino acids; the transcribed region is contained in three exons separated by two introns or intervening sequences (IVS).The first exon encodes amino acids 1 to 29 together with the first two bases for codon 30, exon 2 encodes part of residue 30 together with amino acids 31 to 104, and exon 3, amino acids 105 to 146. Exon 2 encodes the residues involved in heme binding and αβ dimer formation, while exons 1 and 3 encode for the non-heme-binding regions of the β globin chain. Conserved sequences important for gene function are found in the 5' promoter region, at the exon-intron junctions, and in the 3' untranslated region (3'-UTR) at the end of the mRNA sequences. The β globin gene promoter includes 3 positive cis-acting elements: TATA box (positions –28 to –31), a CCAAT box (positions –72 to –76) and duplicated CACCC motifs (proximal at positions –86 to –90, and distal at position –101 to –105). While the CCAAT and TATA elements are

found in many eukaryotic promoters, the CACCC sequence is found predominantly in erythroid cell-specific promoters. Binding of the Erythroid Krüppel Like Factor (EKLF) to the CACCC motif appears to be crucial for normal adult β globin expression.[8,9] In addition to these motifs, the region upstream of the β globin promoter contains two binding motifs for the erythroid transcription factor GATA 1. The importance of these various 5' flanking sequences for normal gene expression is underscored by β thalassemia arising from point mutations in these sequences specifically in and around the TATA box and the CACCC motifs in the –80 to –100 region. An enhancer is also found in intron 2 and 3' of the globin gene, 600 to 900 bp downstream of the poly (A) site.[10]

The 5' untranslated region (UTR) occupies a region of 50 nucleotides between the CAP site, the start of transcription, and the initiation (ATG) codon. There are two prominently conserved sequences in the 5' UTR of the various globin genes (both α and β). One is the CTTCTG hexanucleotide found 8 to 13 nucleotides downstream from the CAP site, i.e. at positions +8 to +13. The second conserved sequence is CACCATG, in which the last three nucleotides form the initiation codon (ATG). Again, the importance of these sequences in the regulation of β gene expression is exemplified by the several mutations in the 5' UTR causing β thalassemia.

The 3' UTR constitutes the region between the termination codon (TAA) and the poly (A) tail. It consists of 132 nucleotides with one conserved sequence, AATAAA, located 20 nucleotides upstream of the poly (A) tail. This consensus hexanucleotide serves as a signal for the cleavage of the 3' end of the primary transcript and addition of a poly (A) tract which confers stability to the processed mRNA and enhances translation. Several mutations affecting the AATAAA sequence and other sequences in the 3' UTR causing β thalassemia have been described. The β-like genes undergo two *switches* (embryonic → fetal → adult). At 6 months after birth, Hb F comprises less than 5% of the total hemoglobin and continues to fall until reaching the adult level of <1% at 2 years of age. It is at this stage that mutations affecting the β gene become clinically apparent. The *switch* from fetal (γ) to adult (β) hemoglobin production is not complete since small amounts of β expression persist in adult life. The residual amount of fetal hemoglobin ($\alpha_2\gamma_2$) is present in a sub-set of erythrocytes called F cells which also contain adult ($\alpha_2\beta_2$) hemoglobin. The tissue- and developmental- specific expression of the individual globin genes is governed by the direct physical interactions between the globin promoters and the βLCR,[11,12] the interaction is mediated through binding of tissue-restricted and ubiquitous transcription factors. This developmental expression is thought to rely on two mechanisms, gene silencing and gene competition, mediated by the different transcription factors in embryonic, fetal and adult cells. While the ε- and γ globin genes are autonomously silenced at the appropriate developmental stage, expression of the adult β globin gene depends on lack of competition from the γ gene for the LCR sequences. This is supported by the down-regulation of the *cis* β gene when γ gene is up regulated by point mutations in their promoters as illustrated by the non-deletional hereditary persistence of fetal hemoglobin (HPFH).[13] Mutations which affect the β promoter, which remove competition for the βLCR also, tend to be associated with variable increases in γ and δ gene expression (*see later*).

Molecular basis of β thalassemia

There are two main varieties of β thalassemia alleles; β^0 thalassemia in which no β globin is produced, and β^+ thalassemia in which some β globin is produced, but less than normal. Less severe forms are sometimes designated β^{++} to reflect the minimal deficit in β chain production. Although more than 200 β thalassemia alleles have been characterized (*HbVar, available at URL http://globin.cse.psu.edu/globin/hbvar/*), population studies indicate that about 40 account for 90% or more of the β thalassemias worldwide.[4] This is because in the areas in which it is prevalent, only a few[4] mutations are common, with a varying number of rare ones, and each of these populations has its own spectrum of β thalassemia alleles.

In contrast to the α thalassemias, the β thalassemias are rarely caused by deletions.[7,14] One group of deletions affects only the β globin gene and ranges in size from 290 bp to >60 Kb. Of these, only the 619 bp deletion at the 3' end of the β gene is common, but even that is restricted to the Sind and Punjab populations of India and Pakistan where it accounts for ~20% of the β thalassemia alleles.[15,16] The other deletions, although extremely rare, are of particular functional and phenotypic interest because they are associated with unusually high levels of Hb A_2 in heterozygotes. These deletions differ widely in size, but remove in common a region from positions –125 to +78 relative to the mRNA cap site in the β promoter which includes the CACCC, CCAAT and TATA elements. The mechanism underlying the markedly elevated levels of Hb A2 appears to be related to the removal of the 5' promoter region of the β gene. This may remove competition for the upstream LCR leading to its increased interaction with the γ and δ genes in *cis*, enhancing their expression.

Indeed, studies of an individual heterozygous for the 1.39 Kb deletion and a δ chain variant showed that there is a disproportionate increase of variant Hb A_2 ($\alpha_2\delta_2$) derived from the δ globin gene *cis* to the β globin gene deletion.[17] This mechanism may also explain the

moderate increases in Hb F which characterize this group of deletions and those due to point mutations affecting the promoter region. Although the increases in Hb F are variable, and modest in heterozygotes, they are adequate to compensate for the complete absence of β globin in homozygotes.[18,19] Two homozygotes for different deletions of this kind have a mild disease despite the complete absence of Hb A_2 ($\alpha_2\beta_2$).

Expression of the β globin gene can also be silenced by deletions of the β globin complex[1,20] as part of $(\varepsilon\gamma\delta\beta)^0$ thalassemia. At a molecular level, the deletions causing $(\varepsilon\gamma\delta\beta)^0$ thalassemia fall into two sub-groups; one group removes all or a greater part of the β globin gene complex including the β gene itself. The other sub-group of deletions removes extensive upstream regions leaving the β globin gene intact, although its expression is silenced because of inactivation of the βLCR. Heterozygotes for such $(\varepsilon\gamma\delta\beta)^0$ thalassemia have a blood picture typical of β thalassemia trait but with a normal Hb A_2 level.

The vast majority of β thalassemias are caused by point mutations within the gene or its immediate flanking sequences. These single base substitutions, minor insertions or deletions of a few bases are classified according to the mechanism by which they affect gene regulation: transcription, RNA processing or RNA translation.[14] Mutations affecting transcription can involve either the conserved DNA sequences that form the β globin promoter or the stretch of 50 nucleotides in the 5'UTR. Generally they result in a mild to minimal deficit of β globin output and can be *silent* in carriers (*see below*).

Mutations that affect RNA processing can involve either of the invariant dinucleotides (GT at 5' and AG at 3') in the splice junction in which case normal splicing is completely abolished with the resulting phenotype of β^0 thalassemia. Mutations within the consensus sequences at the splice junctions reduce the efficiency of normal splicing to varying degrees and produce a β^+ phenotype that ranges from mild to severe. Mutations within introns or exons might also affect the splicing pattern of the pre-mRNA. For example, a cryptic splice site which contains the sequence GT GGT GAG G has been found in exon 1 of the β globin gene, spanning codons 24 to 27. Three mutation within this region activate this cryptic site which acts as an alternative donor site in RNA processing. The mutation in codon 26 (GAC→AAE) that gives rise to Hb E (β26 Gln→Lys) is one such mutation that activates this cryptic splice site, with a reduction of the normal splicing that produces the Hb E variant. Since Hb E production is also quantitatively reduced, the compound heterozygous state, Hb E/β thalassemia, results in a clinical picture closely resembling homozygous β thalassemia ranging from severe anemia and transfusion-dependency to thalassemia intermedia. Other RNA processing mutants affect the polyadenylation signal (AATAAA) and the 3'UTR. These are generally mild β^+ thalassemia alleles.

Approximately half of the β thalassemia alleles affect the different stages of RNA translation and in all instances, no β globin is produced resulting in β^0 thalassemia. Most of these defects result from the introduction of premature termination codons due to frameshifts or nonsense mutations and nearly all terminate within exon 1 and 2. Mutations that result in premature termination early in the sequence (in exons 1 and 2) are associated with minimal steady-state levels of β mRNA in erythroid cells, due to an accelerated decay of the abnormal mRNA referred to as nonsense-mediated mRNA decay (NMD).[21] In heterozygotes for such cases, no β chain is produced from the mutant allele and only half the normal β globin is present, resulting in a typical asymptomatic phenotype. By contrast, mutations that produce in-phase termination later in the β sequence, in exon 3 are not subjected to NMD, resulting in substantial amounts of abnormal β mRNA comparable to that of the normal allele.[22,23] Heterozygotes for such late termination mutations tend to have a much more severe phenotype, and the mutations are termed dominantly inherited (*see below*). Other mutations of RNA translation affect the initiation (ATG) codon and again these result in β^0 thalassemia.

Variants of β thalassemia

Dominantly inherited β thalassemia

In contrast to the common β thalassemia alleles that are prevalent in malarial regions and inherited typically as Mendelian recessives, some forms of β thalassemia are dominantly inherited, in that inheritance of a single β thalassemia allele results in a clinically detectable disease despite a normal α globin genotype.[2,24] Heterozygotes have a thalassemia intermedia phenotype with moderate anemia, splenomegaly and a thalassemic blood picture. Apart from the usual features of heterozygous β thalassemia, such as increased levels of HbA_2 and the imbalanced α/β globin biosynthesis, large inclusion bodies similar to those seen in thalassemia major are often observed in the red cell precursors, hence the original term of *inclusion body thalassemia*.[25] More than 30 dominantly inherited β thalassemia alleles have now been described;[2,24] they include a spectrum of molecular lesions from missense mutations to truncated β variants resulting from nonsense mutations. The common denominator of these mutations is the predicted synthesis of highly unstable β chain variants, so unstable that in many cases, they are not detectable and only implied from the DNA sequence. The predicted synthesis is supported by the presence of substantial

amounts of abnormal β mRNA in the peripheral reticulocytes,[23] in amounts comparable to that produced from the normal β allele. Indeed, the large intra-erythroblastic inclusions, that are so characteristic of this form of β thalassemia, have subsequently been shown to be composed of both α and β globin chains.[26] In contrast, the inclusion bodies in homozygous β thalassemia consist only of precipitated α globin. The differential effects of these in-phase termination mutants on the accumulation of mutant mRNA exemplify how shifting the position of a nonsense codon can alter the phenotype of recessive inheritance caused by haplo-insufficiency, to a dominant negative effect due to the synthesis of an abnormal and deleterious protein.

Again, a spectrum in phenotypic severity in this class of β thalassemia variants is observed, which can be related to a variation in the degree of instability of the β globin products. The phenotype resembles the intermediate forms of β thalassemia by virtue of the ineffective erythropoiesis, but there is also a variable degree of peripheral hemolysis. Unlike recessive β thalassemia which is prevalent in malaria-endemic regions, dominant β thalassemias are rare, occurring in dispersed geographical regions where the gene frequency for β thalassemia is very low. The vast majority of the dominant β thalassemia alleles have been described in single families, many as *de novo* events. It is likely that the low frequency of the dominant β thalassemia alleles is due to the lack of positive selection that occurs in the recessive forms. Clinically, since spontaneous mutations are common in dominant β thalassemia, it is important that the disorder should be suspected in any patient with a thalassemia intermedia phenotype even if both parents are hematologically normal and the patient is from an ethnic background where β thalassemia is rare.

Normal Hb A2 β thalassemias

The diagnostic feature of β thalassemia is the hypochromic microcytic red cells and an elevated level of Hb A2 in heterozygotes, whether β^+ or β^0. Normal Hb A2 β thalassemias (previously referred to as type 2) refer to the forms in which the blood picture is typical of heterozygous β thalassemia except for the normal levels of Hb A_2. Most cases result from co-inheritance of δ thalassemia (δ^0 or δ^+) in *cis* or *trans* to the β thalassemia gene, which can be of the β^0 or β^+ type. The $\delta^0$59 (59 A) mutation has been reported to occur in *cis* to the $\beta^0$39 and β^+ IVS1-110 mutations;[27] and δ^+27 (G→T) has been reported to occur in *cis* and in *trans* to the IVS2-745 mutations.[28,29] One relatively common form of normal Hb A2 thalassemia is that associated with $Hb^{Knossos}$ (β27 Ala→Ser). Like Hb E, the mutation β27(GCC→TCC) activates an alternative splice site reducing the amount of normal transcript that contains the variant. Unlike Hb E, the Hb A2 level is not elevated in heterozygotes. The molecular basis for the normal levels of Hb A2 is a δ^0 thalassemia (Cd59-A) *cis* to the β27 Ala→Ser mutation.[30] β haplotype analysis suggests that the $\delta^0$59/$\beta^{Knossos}$ allele is relatively common in the Middle East and Mediterranean.

Another fairly common cause of normal Hb A2 β thalassemia phenotype in the Greek population is the Corfu form of δβ thalassemia, a 7.2 Kb deletion that includes the 5' part of the δ gene.[31,32] Although the *cis* β globin gene is intact, it is down-regulated by a G→A mutation in position 5 of the IVS1. Heterozygotes for the Corfu deletion have a δβ thalassemia phenotype characterized by a variable increase in Hb F and low to normal Hb A2 levels, while homozygotes have almost 100% Hb F with no Hb A2 and trace levels of Hb A. The mutation has been described as separate lesions in two different populations. The normal β gene in *cis* to the 7.2 Kb deletion in an Italian individual is expressed at normal levels[33] while Algerian homozygotes for the βIVS1 5 G→A mutation have a severe transfusion-dependent anemia.[34] The phenotype of normal Hb A2β thalassemia is also seen in heterozygotes for εγδβ thalassemia and overlaps the phenotypes encountered in carriers of α thalassemia.

Silent β thalassaemia

The *silent* β thalassemias cause only a minimal deficit of β globin production. Heterozygotes do not have any evident hematologic phenotype; the only abnormality being a mild imbalance of globin chain synthesis. It is not surprising therefore, that these mutations have been identified in homozygotes who have a typical β thalassemia trait phenotype[35] or in the compound heterozygous state with a severe β thalassemia allele where they cause thalassemia intermedia.[36] *Silent* β thalassemia alleles are not common except for the C→T mutation at position –101 of the β globin gene[37] which accounts for most of the milder forms of β thalassemia in the Mediterranean.[38] Recently, a novel mutation, a C→G transversion, has been reported in the same position.[39] Some of the promoter mutations in the 5' and 3' UTRs are also *silent*. It has been suggested that the $[TA]_x[T]_y$ sequence variation at position –530 of the β globin gene may be responsible for some *silent* β thalassemia carriers.[40] The reduced β globin expression has been related to increased binding of a repressor protein (BP1) isolated from K562 cells.[41] However, population surveys and clinical studies do not show a consistent correlation between the +ATA, T variant and a β thalassemia phenotype, suggesting that it is a neutral polymorphism.[42]

β thalassemia trait with unusually high Hb A_2

Despite the vast heterogeneity of mutations, the increased levels of Hb A_2 observed in heterozygotes for the different β thalassemia alleles in different ethnic groups are remarkably uniform, usually 3.5-5.5% and

rarely exceeding 6%. Unusually high levels of Hb A_2 over 6.5% seem to characterize the sub-group of β thalassemias caused by deletions that remove the regulatory elements in the β promoter. As discussed earlier, the unusually high Hb A2, often accompanied by modest increases in Hb F, may be related to the removal of competition for the upstream LCR, allowing an increased interaction with the *cis* δ and γ genes.

β thalassemia due to insertion of a transposable element

Transposable elements may occasionally disrupt human genes and result in their inactivation. The insertion of such an element, a retrotransposon of the family called L1 has been reported with the phenotype of $β^+$ thalassemia. Despite the insertion of 6-7 Kb DNA into its IVS2, the affected gene expresses full length β globin transcripts at a level corresponding to about 15% of normal β globin mRNA.[43]

β thalassemia due to trans-acting determinants

Population studies have shown that ~1% of the β thalassemias remain uncharacterized despite extensive sequence analysis, including the flanking regions of the β globin genes. In several families, linkage studies demonstrated that the β thalassemia phenotype aggregates independently of the β globin complex implying that the genetic determinant is *trans*-acting.[14,44] Recently, it has been found that mutations in XPD that cause trichothiodystrophy (TTD) are frequently associated with a phenotype of β thalassemia trait,[45] supported by reduced levels of β globin synthesis and reduced β globin mRNA. The XPD protein is a subunit of the general transcription factor TF11H which is involved in basal transcription and DNA repair. Mutations in the transcription factor GATA 1 on the X chromosome have also been reported to cause β thalassaemia in association with thrombocytopenia.[46] This was the first example of β thalassemia in humans caused by a mutation in the erythroid-specific transcription factor.

Somatic deletion of β globin gene

This novel mechanism was recently described in an individual who had moderately severe thalassemia intermedia despite being constitutionally heterozygous for $β^0$ thalassemia with a normal α genotype.[47] Subsequent investigations revealed that he had a somatic deletion of a region of chromosome 11p15 including the β globin complex giving rise to a mosaic of cells, 50% with one and 50% without any β globin gene. The sum total of the β globin product is ~25% less than the normally asymptomatic β thalassemia trait. Subsequently, two unrelated Italian patients were also reported to have thalassemia intermedia caused by somatic deletions of chromosome 11p15 in a subpopulation of hematopoietic cells.[48] These unusual cases once again illustrate that the severity of anemia of β thalassemia reflects the quantitative deficiency of β globin chain production. Furthermore, with respect to potential gene therapy, expression of a single β globin gene in a proportion of the red blood cells appears to be sufficient to redress the chain imbalance to produce a condition mild enough not to need major medical intervention.

Clinical and hematologic phenotypes, pathophysiology and modulating factors of β thalassemia

The clinical manifestations of β thalassemia are extremely diverse, spanning a broad spectrum from the transfusion-dependent state of thalassemia major to the asymptomatic state of thalassemia trait. The most severe end of the spectrum is characterized by the complete absence of β globin production and results from the inheritance of two $β^0$ thalassemia alleles, homozygous or compound heterozygous states. This condition is referred to as β thalassemia major and, at their worst, the patients present within 6 months of life with profound anemia, and if not treated with regular blood transfusions, die within their first two years. β thalassemia trait forms the other end of the phenotypic spectrum of β thalassemia, and is typically associated with the inheritance of a single β thalassemia allele. Carriers for β thalassemia, whether $β^0$ or $β^+$, are clinically asymptomatic; they may have mild anemia with characteristic hypochromic microcytic red blood cells, elevated levels of Hb A2 and variable increases of Hb F (up to 2%). However, as seen earlier, the heterozygous state for β thalassemia can also be extremely diverse, ranging from one that is completely phenotypically *silent*, to one with severe anemia (dominantly inherited).

Between the two extremes of the heterozygous states, severity of the different β thalassemia alleles is reflected in their hematologic phenotype, in the degree of hypochromia and microcytosis as indicated by the mean cell hemoglobin (MCH) and mean cell volume (MCV) values, respectively. Rund *et al.*[49] showed that the $β^0$ thalassemia alleles which are associated with the most severe phenotype demonstrated a fairly tight range of MCV (63.1 fL, SD = 3.4) while the $β^+$ alleles were associated with a wider range of MCV (69.3 fL, SD = 5.6). The cut-off point between the $β^0$ and $β^+$ thalassemias was 67 fL. The broader range of MCV in $β^+$ thalassemia, when compared to $β^0$ thalassemia, is not surprising given the broad range in the deficit of β globin production, from barely detectable levels at the severe end, to just a little less than normal in the very mild or *silent* alleles.

A more recent study has taken the correlation between the severity of β thalassemia alleles with hematologic parameters in heterozygotes to a finer level. Skarmoutsou *et al.*[50] measured a series of hematologic parameters, including reticulocyte hemoglobin content (CHr), soluble transferrin receptor (sTfR), reticulocytes and Hb A_2 and F levels in 57 iron-replete individuals with heterozygous β thalassemia. There was a negative correlation between the values of sTfR, a reliable quantitative assessment of erythropoietic activity, and the severity of the β thalassemia alleles; the values were lowest in the very mild β thalassemia (β silent), and highest in β^0 thalassemia heterozygotes. CHr, a product of reticulocyte hemoglobin and volume, was significantly higher in the β silent group (27.0–32.0 pg) than in severe groups (β^+ and β^0 thalassemia alleles at 19.5–25.3 pg). Furthermore, while sTfR values showed a positive correlation with Hb A_2, there was a significant negative correlation between CHr and Hb A_2 levels. This study confirms that all heterozygous β thalassemias have some degree of ineffective erythropoiesis that varies with the severity of the β thalassemia mutation.

Between the two clinical extremes of thalassemia major and trait, lies the clinical syndrome of thalassemia intermedia which comprises a diverse spectrum of phenotypes, from a condition that is slightly less severe than transfusion-dependence to one that is asymptomatic and often identified through a routine blood test. Dissecting the mechanisms generating the intermediate status has provided tremendous insights into the genetic basis for the phenotypic diversity of the β thalassemias.[51-54] Although definition of the two extremes of the clinical spectrum of β thalassemia is easy, assigning the severity of the intermediate form can be problematic. Criteria such as age and level of hemoglobin at presentation, transfusion history, and the requirements for intermittent blood transfusion have been used, but these have their inherent limitations and are highly clinician-dependent. Thalassemia intermedia can result from the inheritance of one or two β thalassemia alleles. In a large number of patients, the reduced disease severity can be explained by the inheritance of the milder β thalassemia alleles (β^{++} and *silent*) that allow the production of a significant proportion of β globin chains. A substantial number, however, have β^0 thalassemia, and in such cases, the absence of β globin chains is compensated by an inherent ability to produce fetal hemoglobin (Hb F, $\alpha^2\gamma^2$). Co-inheritance of α thalassemia has very little effect on β^0 thalassemia while individuals with α gene deletions and β^+ thalassemia may have milder disease. Yet other thalassemia patients have inherited only one β thalassemia allele. Most cases of unusually severe heterozygous β thalassemia are due to the co-inheritance of extra α globin while others are due to the nature of the underlying β thalassemia mutation itself (*see Dominantly inherited β thalassemia*). Given the differences in the spectrum of β thalassemia mutations and differences in the frequency of α thalassemia, the relative importance of the different factors would vary accordingly in different population groups. It is important to note that the genotypic factors are not mutually exclusive (Table 1).

The underlying pathophysiology of β thalassemia relates to the degree of globin chain imbalance and the excess of α globin chains.[1,55] The latter aggregate in red cell precursors forming inclusion bodies, causing mechanical damage and premature destruction of the cells in the bone marrow. The ensuing ineffective erythropoiesis results in anemia and intense proliferation and expansion (10 to 30 times) of the bone marrow with resulting skeletal deformities. The red cells that survive to reach the peripheral circulation are also prematurely destroyed in the spleen which becomes enlarged, eventually leading to hypersplenism. Anemia in β thalassemia thus results from a combination of ineffective erythropoiesis, peripheral hemolysis, and an overall reduction in hemoglobin synthesis. Factors which reduce the degree of chain imbalance and the magnitude of α chain excess in the red cell precursors, will have an impact on the phenotype. At the primary level, this is related directly to the nature of the β thalassemia mutation itself. At the secondary level, the severity of globin chain imbalance is influenced by variability at two loci:- α globin and γ globin genes.[56]

In many populations in which β thalassemia is prevalent, α thalassemia also occurs at a high frequency and hence it is not uncommon to co-inherit both conditions. As with β thalassemia, the different α thalassemias which predominate in different racial groups display a wide range of severity. This interaction alone provides the basis for considerable clinical heterogeneity; the degree of amelioration depends on the severity of the β thalassemia alleles and the number of functional α globin genes.[51,53] Co-inheritance of a single α gene deletion has very little effect on β^0 thalassemia while individuals with two α gene deletions and β^+ thalassemia may have milder anemia. At the other extreme, patients who have co-inherited Hb H disease (equivalent to only one functioning α gene) and homozygous β thalassemia, also have moderately severe anemia.[57]

In β thalassemia heterozygotes, co-inheritance of α thalassemia normalizes the hypochromic microcytosis,[58] while the presence of increased α globin product in β thalassemia heterozygotes tips the globin chain imbalance further, converting a typically clinically asymptomatic state into that of thalassemia intermedia. In the majority of cases, this is related to the co-inheritance of triplicated α globin genes. Triplicated α genes (ααα/) occur in most populations at a low fre-

Table 1. Molecular basis of thalassemia intermedia.

Homozygous or compound heterozygous state for β thalassemia
Inheritance of mild β thalassemia alleles
Co-inheritance of α thalassemia
Increased Hb F response
Xmn1 ^{G}γ polymorphism
β globin gene promoter mutations
Trans-acting HPFH genetic determinants
Heterozygous state for β thalassemia
Co-inheritance of extra α globin genes
(ααα/αα, ααα/ααα, αααα/αα)
Dominantly inherited β thalassemia
(Hyperunstable β globin chain variants)
Compound heterozygotes for β thalassemia and β chain variants
e.g. Hb E/β thalassemia
Compound heterozygotes for β thalassemia and HPFH or δβ thalassemia

quency. The co-inheritance of two extra α globin genes (ααα/ααα) or αααα/αα) with heterozygous β thalassemia results in thalassemia intermedia.[59,60] However, the phenotype of a single extra α gene (ααα/αα) with heterozygous β thalassemia is more variable and depends on the severity of the β thalassemia allele.[61,62] There appears to be a critical threshold of globin chain imbalance in each individual above which clinical symptoms appear.

Globin chain imbalance can also be reduced if there is an inherent capacity for producing γ chain which combines with the excess α to form fetal hemoglobin (Hb F, $\alpha_2\gamma_2$). Although the production of Hb F is almost switched off at birth, all adults continue to produce residual amounts of Hb F. In all β thalassemias, Hb F levels are relatively increased due to the selective survival of the erythroid precursors that synthesize relatively more γ chains. However, patients with β thalassemia differ considerably in their ability to synthesize γ chains and their Hb F response. This becomes evident in the group of homozygous β^0 thalassemia patients who have a mild disease despite the absence of Hb A.[51,54] These patients appear to have an inherited ability to produce Hb F and are able to maintain a reasonable level of hemoglobin, all of which is Hb F. Hence, against this background of an increase from the expanded erythroid mass and the selective survival of F cells, are genetic factors which account for the individual Hb F response to the *stress* of β thalassemia.

Genetic determinants influencing Hb F response can be within the β globin complex or *trans*-acting. The C→T substitution at position –158 of the Gγ globin gene, referred to as the *Xmn*1-Gγ polymorphism, is a common sequence variant in all population groups, present at a frequency of 0.32 to 0.35.[63] Our studies show that this genetic variant accounts for about one third of the variation in Hb F levels in normal adults.[63] Although the increases in Hb F and F cells are minimal in normal people, clinical studies have shown that, under conditions of hematopoietic stress, for example in homozygous β thalassemia and sickle cell disease, the presence of the *Xmn*1-Gγ site favors a higher Hb F response.[64,65] This could explain why the same mutations on different β chromosomal backgrounds (some with and others without the *Xmn*1-Gγ site) are associated with disease of different clinical severity.

Other determinants within the cluster are related to the mutation itself. The increased Hb F output observed in deletions or mutations that involve the promoter sequence of the β globin gene reflect the competition between the γ and β globin gene promoters for interaction with the LCR. Hence, although such deletions cause a complete absence of β globin product, the severity of the phenotype is offset by the concomitant increase in hemoglobin F.[18]

Although the presence of the cis *Xmn*1-Gγ site is a modulating factor, clearly there are some patients who have an enhanced Hb F response despite being *Xmn*1-Gγ -/-.[51,54] In many cases, family studies have shown this inherent capacity for producing Hb F is due to a genetic determinant that is not linked to the β globin cluster. This is in keeping with our sib-pair studies in normal adults which showed that >50% of the F cell variance in the general population is accounted for by *trans*-acting factors.[63] Indeed, analysis of a group of thalassemia intermedia patients revealed seven sib-ships with discordant phenotypes despite identical α and β genotypes. The steady state Hb F values between the siblings ranged from 1 g/dL to as much as 8/9 g/dL and was ascribed to genetic determinants not linked to the β globin complex.[51] *Trans*-acting loci controlling Hb F and F cell levels have now been mapped to three regions of the genome – chromosomes 6q23, Xp22 and 8q.[66–68] As the genetic basis of the propensity to produce Hb F becomes unravelled it is becoming clear that the conglomeration of the *Xmn*1-Gγ polymorphism, the QTL on 6q, Xp and 8q and others, linked and unlinked to the β globin complex, constitute the loosely defined syndrome of heterocellular HPFH.[13] These *trans*-acting factors presumably play an important role in the fine tuning of γ globin production in normal adults, in the response to *erythropoietic stress* and possibly, in the capacity to respond to pharmacologic inducers of Hb F synthesis. But until the molecular basis of these different entities is characterized, detection of an inherent capacity for increased Hb F production is, at present, difficult and usually inferred from family studies. Apart from the number of α globin genes and an inherent capacity to produce Hb F, the proteolytic capacity of the erythroid precursors in catabolising the excess α globin chains has often been suggested as another factor but this effect is difficult to define. Recently, the newly discovered α hemoglobin stabiliz-

ing protein (AHSP), a chaperone of α globin,[69] has been suggested as another genetic modifier, but so far studies have been inconclusive.[70,71]

Secondary complications of β thalassemia and genetic modifiers

With the increasing lifespan of the β thalassemia patients, subtle variations in the phenotype with regard to some of the complications in the older patients have become apparent and evidence suggests that they may be affected by genetic variants.

Bilirubin levels and gallstones

Hyperbilirubinemia and a propensity to gallstone formation is a common complication of β thalassemia and is attributed to the rapid turn-over of the red blood cells, bilirubin being a break-down product of hemoglobin. Studies have shown that the levels of bilirubin and the incidence of gallstones in β thalassemia, from trait to major,[72-74] is related to a polymorphic variant (seven TA repeats) in the promoter of the uridine diphosphate-glucoronyltransferase IA (*UGTIA*) gene, also referred to as Gilbert's syndrome. *In vitro* studies indicate that the variant reduces expression of the *UGTIA* gene.[75] Normal individuals who are homozygous for the $[TA]_7$ variant instead of the usual six, tend to have higher levels of bilirubin.[75] The $[TA]_7$ variant has also been shown to be associated with increased bilirubin levels in sickle cell disease[76,77] and other hemolytic anemias.

Iron loading

A common complication of β thalassemia involves organ damage from iron overload, not just from blood transfusions but also from increased absorption. In a patient with thalassemia intermedia, co-inheritance of a single copy of the C282Y mutation in the *HFE* gene was associated with hemochromatosis and diabetes,[78] while the co-existence of β thalassemia trait aggravates and accentuates iron loading in C282Y *HFE* homozygotes.[79] Since the C282Y mutation is rare in populations in which β thalassemia is common it has a limited role in iron loading amongst these patients.[80] Much more common is the H63D polymorphism in the *HFE* gene, whose functional role is still being investigated. A recent study showed that β thalassemia carriers who are homozygous for H63D in the *HFE* gene have higher serum ferritin levels than carriers without the polymorphism, suggesting that the H63D polymorphism may have a modulating effect on iron absorption.[81] As other genes in iron homeostasis become uncovered, it is likely that genetic variants will be found in these loci that influence the different degrees of iron loading in β thalassemia.[82,83]

Bone disease

Progressive osteoporosis and osteopenia is another increasingly common complication encountered in young adults with β thalassemia.[84,85] Several studies suggest that the prevalence of bone disease in β thalassemia is higher in men than in women, and that it is more severe in the spine than in the femoral neck.[86-88] It is manifested by diffuse bone pain, particularly in the lower back, vertebral fractures, cord compression, spontaneous fractures and femoral head necrosis. Bone mass is determined by a combination of genetic and environmental factors; anemia and bone marrow expansion which are prevalent in β thalassemia are major contributors in inadequately treated patients. The osteoporosis that occurs in other patients who are reasonably well transfused but in whom there is severe iron loading, may be related to hypogonadism.[89] However, it has become apparent from recent studies that bone disease is increasingly common, with a frequency of 40-50%,[84] even among the well-transfused and iron-chelated patients which may be related to the prolonged use of desferrioxamine. Further, some patients appear to be more susceptible to bone disease than others. Bone mass is a quantitative trait influenced by several quantitative trait loci (QTL)[90-92] including the estrogen receptor gene, the vitamin D receptor (*VDR*), *COL1A1* and *COL1A2* genes, and transforming growth factor β1 gene (*TGFB1*). Polymorphism in *TGFB1* has been associated with severe osteoporosis and increased bone turnover in women[93] but a study of *TGFB1* polymorphism failed to demonstrate a statistical difference in β thalassemia patients with different bone mineral densities. The VDR gene polymorphism in intron 8 (involving the *Bsm1* site) was associated with osteopenia in thalassemia.[88] A G→T polymorphism involving an Sp1 binding site in the collagen type α1 gene (*COL1A1*) is strongly associated with reduced bone mass and osteoporosis.[94] This same polymorphism in the *COL1A1* gene has also been shown to be strongly associated with reduced bone mineral density and osteoporosis in β thalassemia.[87]

Cardiac disease

Cardiac complications are the main cause of death in β thalassemia. Many aspects of cardiac complications are still poorly understood, and again it is clear that cardiac disease in β thalassemia is multifactorial reflecting the chronic anemia, iron overload and pulmonary hypertension. The situation is further confounded by the unpredictability of the severity of cardiac iron deposition. A risk factor for left ventricular failure in thalassemia is decreased anti-oxidant activity of apolipoprotein (APOE) ε4 related to the frequency of the apolipoprotein ε4 allele.[95] More recently, the APOE ε4 allele has also been shown to be an independent risk factor for cardiac dysfunction in elderly people.[96] The

risk of cardiac dysfunction was increased 3-fold in individuals homozygous for APOE ε4.

Other complications

Genetic variants implicated in other complications of β thalassemia include: specific HLA alleles in the tendency to develop hepatitis and liver cirrhosis; genetic variants in factor V, prothrombin and MTHFR, and the tendency to develop thrombosis. Eldor and Rachmilewitz[97] presented compelling evidence for an increased risk of thrombosis in the different thalassemias and α thalassemia syndromes. A retrospective review by Capellini *et al.*[98] showed that thalassemia intermedia patients who had been splenectomized are particularly at risk of developing venous thromboembolic events. Chronic lung disease and pulmonary hypertension are other complications that are being increasingly recognized in older β thalassemic patients, and are thought to be related to the increased tendency to thrombosis[97] and small pulmonary emboli.[99] A mechanism related to the scavenging of nitric oxide by free plasma hemoglobin implicated in sickle chronic lung disease may also underlie the pathogenesis of pulmonary hypertension in β thalassemia.[100]

Conclusions

The genetic heterogeneity underlying the phenotypic diversity of the β thalassemias is prototypical of how the wide spectrum in disease severity of a monogenic disorder can be generated at different levels – severity of anemia at the primary level and severity of complications related to the anemia and to treatment at the secondary level. An overview of the molecular basis of β thalassemia has been presented followed by a short description of the clinical and hematologic diversity, and the underlying pathophysiology. With the increasing lifespan of patients with β thalassemia, it seems likely that an increasing number of complications secondary to this condition will be encountered in the older patients. An outline of the various genetic loci (modifier genes) that modulate the secondary complications affecting various organs has been presented.

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